DOCKET NO.: GIES3002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF	: Michael Giesing and Bernhard Suchy
SERIAL NO.	: 10/525,019
FILED	: June 28, 2005
FOR	: Method for analyzing body fluids for the presence of cancer cells, use thereof, corresponding analysis kits, and use of specific active substances for treating cancer

DECLARATION UNDER 37 C.F.R. §1.132

COMMISSIONER OF PATENTS
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SIR:

Now comes Prof. Dr. med. Michael Giesing who deposes and states:

1. I am a graduate of University of Bonn, Germany, and received my doctorate degree in the year 1971.

2. I have been working for 14 years as a laboratory physician in the field of molecular oncology.

3. I have read and fully understood U.S. application, Ser. No. 10/525,019.

4. I have read and fully understood the Office Action of May 15, 2008 and the references cited therein.

5. The following experiments and investigations were carried out by me or under my direct supervision.

For more than a decade I have been using the molecular characterization of disseminated cancer cells as a diagnostic tool that allows an "online approach" to cancer management.

Serum PSA is the landmark protein for the detection of prostate cancer. The limited specificity of tPSA between 4 to 10 ng/ml prompted me to study whether the molecular PCR-based detection of disseminated cancer cells can be used in the early diagnosis of prostate tumors and in monitoring disease progression to determine the relapse-free survival and the risk of developing a metastasis or a recurrence.

A total of 240 patients were enrolled in the study after giving informed consent. The patients were recruited by more than 30 urologists in their offices and by clinicians respectively. Patients were devided into two main groups. In the presurgery group, 129 patients with a serum PSA level between 4 and 10 ng/ml were enrolled for molecular testing of circulating prostate cells. Although a pretest biopsy was not an exclusion criterion 31 patients had a previous negative biopsy. After molecular testing additional 7 patients were excluded from further evaluation because they were reluctant to undergoe clinical diagnosis. Posttest clinical diagnosis was performed through biopsy and in a subgroup of 12 patients in one center by ^{11}C-choline PET followed by surgery. MRT was applied in 1 patient. In the postsurgery group, 111 patients were enrolled. 6 additional patients were excluded from evaluation because of missing clinical data. 77 patients remained without tumor recurrence within the observation time. 34 patients underwent relapse tumors, 14 (41%) with local relapse, 9 (26%) with non regional lymph nodes (M1a), 33 (97%) with bone metastases (M1b), 4 (12%) with metastases in other sites (M1c). Demographic data are listed in Table 1.

Table I. Demographic Data		
	Median	Range
Tumor Diagnosis (n=129) – group A (tumor; n = 42) and B (no tumor; n = 87)		
Age (Yr)	64	41-82
Medium time to diagnosis	6	1-28
Gleason score (group A)	7	6-9

Tumor size (group A)	2	2-4
Nodal Status (group A)	0	0
post surgery (n=111)		
group C (no progression risk; n=77)		
Age (Yr)	65	49-89
Relapse free survival (mo)	31	1-216
Gleason score	7	2-10
Tumor size	2	1-4
Nodal Status	0	0-1
group D (at risk for progression; n=34)		
Age (Yr)	67	57-80
Time to relapse (mo)	16	0-146
Gleason score	7	7-10
Tumor size	3	1-4
Nodal Status	1	0-2

Methodology

Peripheral blood was collected in heparinized Vacutainer systems (Becton Dickinson). If not collected by car service blood specimen were allowed a maximum period of 24 h until processing in the laboratory. Decay studies of circulating cells (qnRT-PCR of CK20 expressing cells) have shown cellular stability for 36h when exposed to various temperatures and shaking (unpublished results).

Mononucleated cells (MNC) were purified over a density gradient using Nycoprep 1.077 (Nycomed, Norway). MNC were washed twice in PBS (0.2% BSA; Life Technologies, Germany) and resuspended finally in 12.2 ml PBS. MNC served for the enrichment of two prostate cell fractions. MNC were rinsed 10 times using 5 ml PBS each. The final volume was 12.2 ml.

Circulating large cancer cells ($\varnothing \geq 20\mu m$) and cell clusters (CCC) derived from 5ml of the final MNC suspension were enriched by size using a column containing a polyester mesh (width 20 μm in diameter). Cells retained on the mesh were lysed with Trizol (Life Technologies, Germany) and processed to DNA analysis.

Total RNA was prepared and processed to reverse transcription as usual. To monitor the efficiency of cDNA synthesis , real time quantitative RT-PCR for glyceraldehyde-3-phosphate dehydrogenease (GAPDH) was performed using a reference cell line and yielding a normalized

cDNA standard curve. PCR amplification products in the presence of target-specific, double-labeled fuorogenic probes were quantitated in real-time using the ABI PRISM 7700 Sequence Detection System (PE Biosystems, USA). Normalized target gene message (Target/GAPDH) is expressed as relative specific expression (RSE) calculated from the ratio of circulating prostate cells and MNC. Normalized reference expression values were calculated as cell equivalents stemming from target enriched reference cell lines and MNC after cell counting in a Müller chamber. Target gene expression was considered positive if the Ct value was at least one log lower compared to controls. Target gene primers and probe designed by using the Primer Express software 1.0 (Perkin-Elmer, USA) were purchased from TIBMOLBIOL, Germany. Primer designs are overspanning two exons without intron sequences thereby avoiding DNA contamination.

For the investigation of CCCs, the following marker-RNA's were chosen:

SOD2 (NM_000636; 6q25)

sense: 5'- GTCACCGAGGAGAAGTACCAGG -3'

antisense: 5'-GGGCTGAGGTTTGTCCAGAA-3'

probe: 5'- CGTTGGCCAAGGGAGATGTTACAGCCC -3'

ref. cell line: EFM192;

TXNRD1 (NM_003330; 12q23-q24.1)

sense: 5'-GGAGGGCAGACTTCAAAAGCTAC -3'

antisense: 5'-ACAAAGTCCAGGACCATCACCT -3'

probe: 5'-TTGGGCTGCCTCCTT AGCAGCTGCCA -3'

ref. cell line: MES

GPX1 (NM_201397; 3p21.3)

sense: : 5'- CTCGGCTTCCCGTGCAA -3'

antisense: 5'-TGAAGTTGGGCTCGAACCC -3'

probe: 5′-GTTTGGGCATCAGGAGAACGCCAAGAA -3′

ref. cell line: EFM192.

Sequence comparison reveals that the following transcript variants will be amplified and detected if the primers and probes described are used.

MNSOD	NM_001024465.1
	NM_000636.2
TXNRD1	NM_001093771.1
	NM_003330.2
	NM_182742.1
	NM_182729.1
	NM_182743.1
GPX1	NM_000581.2
	NM_201397.1

Differential detection of said variants may be achieved with appropriate primer and probe designs.

All data were entered into Microsoft Office Excel spread-sheats. Statistical analyses were performed using Statistica 8.0 software (StatSoft, Germany). Comparison of the molecular tests between patient groups was achieved with the t-test for independent variables. Sensitivity, specificity, PPV, NPV, accuracy, odds ratio (OR) and prevalence were calculated using the 2x2 contingency table method applying the chi-square test for statistical evaluation. Receiver operating characteristic (ROC) analysis and areas under the curve (AUCs) were used as objective measures to evaluate the molecular cell markers. The Hanley-McNeil test was applied for calculations of standard errors. The ROC calculation software was additionally programmed by StatSoft, Germany.

For examination of Gleason Score, tumor size, nodal status and molecular cell markers at the clinical levels given, Spearman`s correlation coefficient by rank was calculated. For postsurgical survival analysis of the relapse-free survival (RFS) Kaplan-Meier curves were used. Patients without relapse tumors were censored. Differences between groups were calculated using

the log-rank test. Univariate and multivariate analysis for comparing patients' tumor size, nodal status, Gleason Score with progression of the disease was done using the Cox proportional hazards model. P-values were derived from Wald's Chi-Square test. For categorical and continuous predictors and classification of cases logistic regression analysis was applied. P-values < 0.05 were considered as statistically significant.

Results

Molecular analysis of CCC and CEC was performed in a total of 240 patients at various clinical stages. Two cohorts have been investigated, the first prior to surgery with (group A) or without tumor (group B), the second after surgery (group C and D). The latter cohort was found to have two subgroups being different in molecular data. The presurgical group has been examined for tumor diagnostic purposes (group A with tumor, group B without tumor), the postsurgical group has been studied for disease prognostication. Group C and D are different with respect to the effects of surgery to reduce the load of CCC.

5.1 Early detection of primary prostate cancer

Figure 1 shows antioxidant gene expression in circulating cell clusters at the tumor diagnostic level (A and B) and after surgery (C and D). Relative specific expression (RSE: cell equivalents of target gene/GAPDH in CCC/patient MNC) is given.

Figure 1:

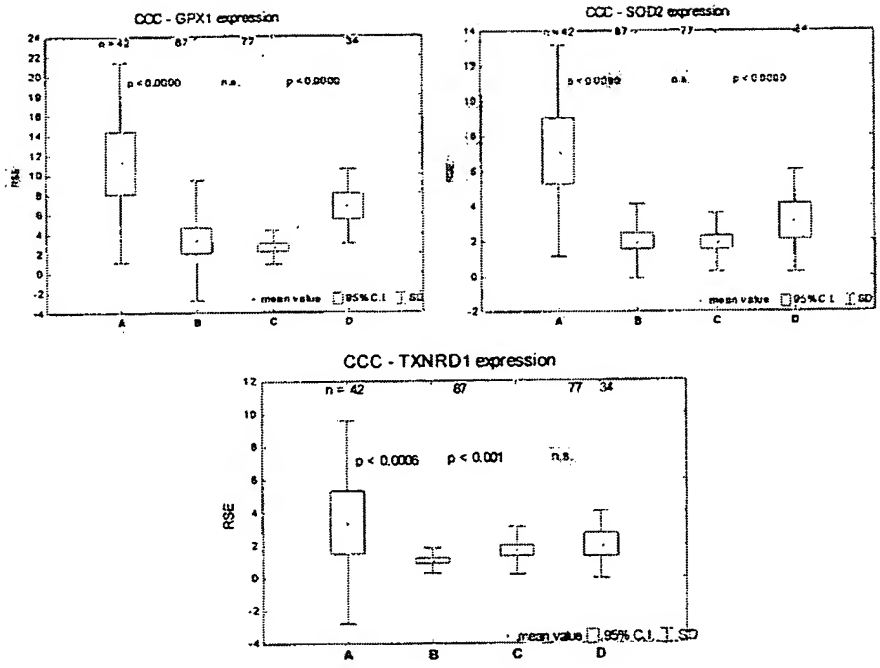
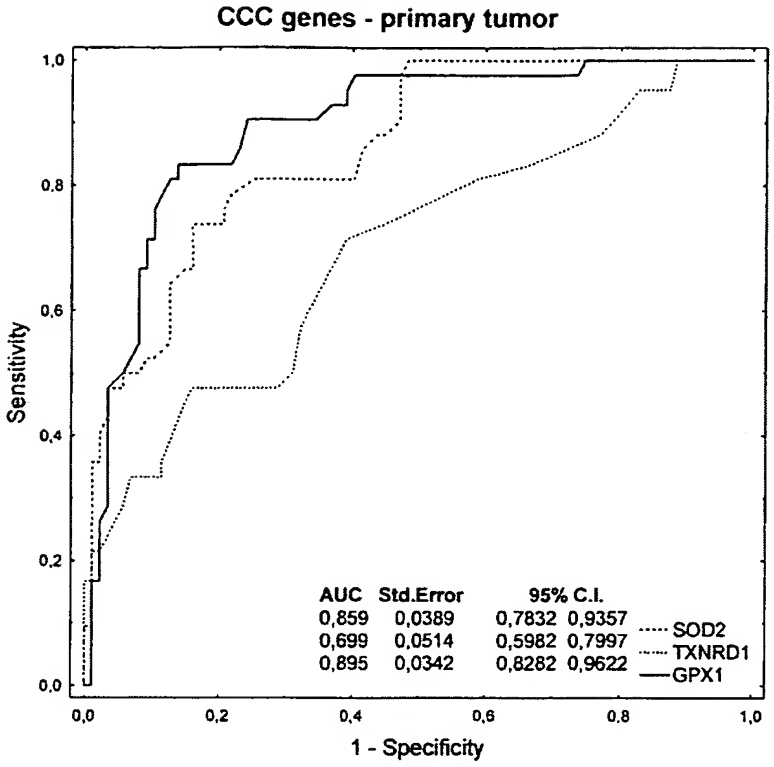


Table II. Performance of predictive gene expression in CCC								
Gene	Sensitivity	Specificity	PPV	NPV	Prevalence	accuracy	odds ratio	p-value
	CCC gene expression (Threshold values: -95% C.I. Group A)							
GPX1	43%	97%	86%	78%	33%	79%	21	0,0000
SOD2	48%	95%	83%	79%	33%	80%	20	0,0000
TXNRD1	48%	78%	51%	76%	33%	70%	3.3	0,0028
all	86%	82%	69%	92%	33%	83%	28	0,0000

Figure 2:



Spearmans Correlation by Rank (pre surgery - tumor vs. non-tumor)

	Valid - N	Spearman - R	t(N-2)	p-Niveau
Diagnosis & SOD2	129	0,583689	8,101007	0,000000
Diagnosis & TXNRD1	129	0,323447	3,852133	0,000185
Diagnosis & GPX1	129	0,641631	9,427239	0,000000

Summary: Parameters and Standard Error

Logistic Regression Analysis (pre surgery - tumor vs. non tumor)

	Konst.B0	SOD2	TXNRD1	GPX1
Estimate	-2,917048	1,546782E+00	-0,7164642	-0,3597689
Standard Error	0,4897262	3,804362E-01	0,1965932	0,1100651
t(125)	-5,956487	4,065813E+00	-3,6444	-3,268693
p-Level	0,0000000243854	8,408503E-05	0,0003914875	0,001395641
-95%CL	-3,886276	7,938520E-01	-1,105546	-0,5776013
+95%CL	-1,947819	2,299713E+00	-0,3273819	-0,1419365
Wald's Chi-quadra	35,47974	1,653084E+01	13,28165	10,68436

p-Level	0,000000002604055	4,797968E-05	0,0002684401	0,001081593
Odds Ratio (per unit)	0,05409316	4,696335E+00	0,4884764	0,6978375
-95%CL	0,02052162	2,211900E+00	0,33103	0,561243
+95%CL	0,1425848	9,971320E+00	0,7208084	0,8676763
Odds Ratio (Range)			0,000000000000285823	0,000000004872266
-95%CL		4,874300E+09	1,53842900E-18	4,51678500E-14
+95%CL			0,000005310274	0,0005255724

Classification of cases (pre surgery - tumor vs. non tumor)

Diagnosis	Progn – no tumor	Progn - tumor	Percent - correct
No tumor	83	4	95,40230
tumor	20	22	52,38095

The average correct prognosis value was 81 %.

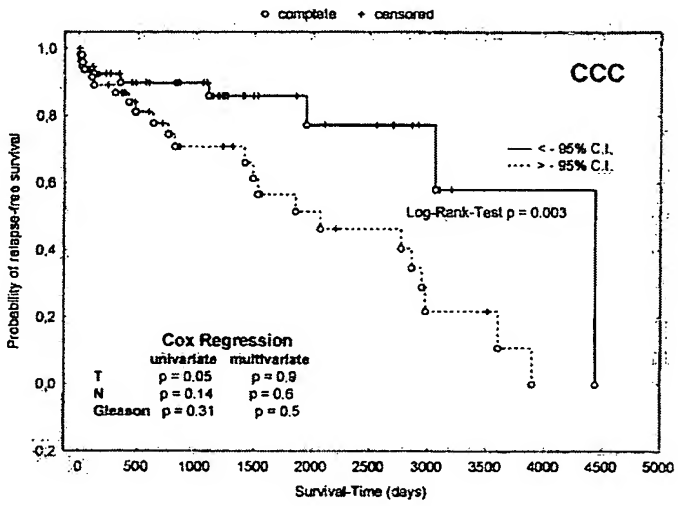
All calculations show that the quantitative measurement of three antioxidant gene expression in circulating cancer cell clusters is a reliable method for the prediction of prostate primary tumor in patients with serum PSA level between 4 and 10 ng/ml. Clearing of these cells by surgery suggests that these cells stem from the tissue whereas incomplete clearance suggests the autonomy of circulating cancer cell clusters.

5.2 Prognosis of the disease course – Relapse-free Survival (RFS)

Which postsurgical group is at risk for disease progression was studied in a prospective fashion. Predictive values were calculated from the -95% CI threshold values of at risk patients (group D). Figure 3 shows that the relapse-free survival for patients overexpressing SOD2 and GPX1 is shorter than for patients not overexpressing SOD2 and GPX1. Both genes are prognosticators of the disease course (relapse-free survival). Multivariate Cox Regression analysis

suggests that SOD2 and GPX1 overexpressing circulating cell clusters function independently from Gleason Score, T and N status of the primary tumor.

Figure 3:

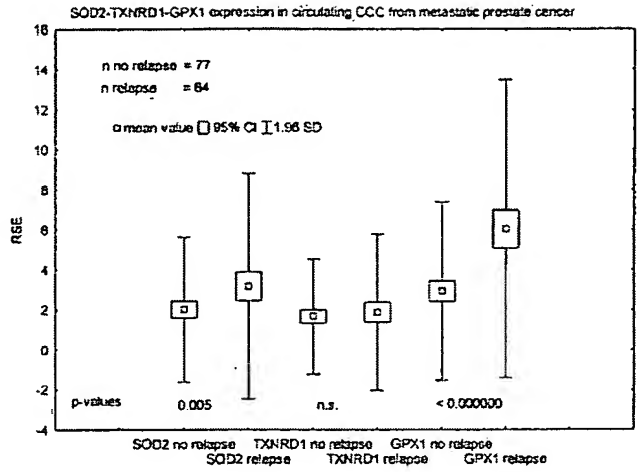


5.3 Prediction of Distant and Local Relapse:

A total of 141 patients (64 patients with local and/or distant relapse and the 77 patients of group C) were enrolled in the assessment.

Figure 4 shows that remarkable overexpression of SOD2 and GPX1 was found in circulating cancer cell clusters throughout the postsurgical period of the disease. Expression remained elevated until relapse tumor formation if no other treatment was given.

Figure 4:



5.3. 1 Distant Relapse

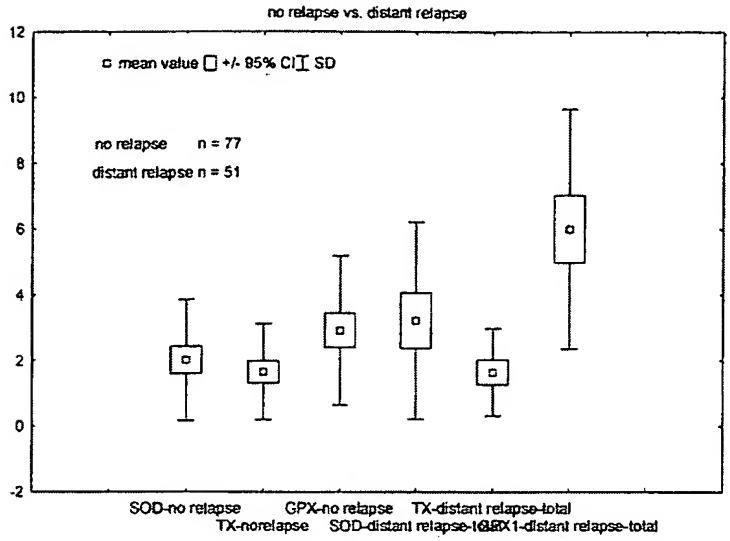
A total of 128 patients (51 patients with distant relapse and the 77 patients without relapse) were enrolled in this assessment.

The genes were assayed at the time of clinical relapse. Predictive values were calculated from the -95% CI threshold values of at risk patients. SOD2 and GPX1 were found to predict distant tumor formation, primarily bone metatstases (figure 5 and 6). The predictive values are given. The performance of SOD2 and GPX1 was excellent as shown in ROC curves, correlation analysis and logistic regression.

Table III Performance of predictive gene expression in CCC

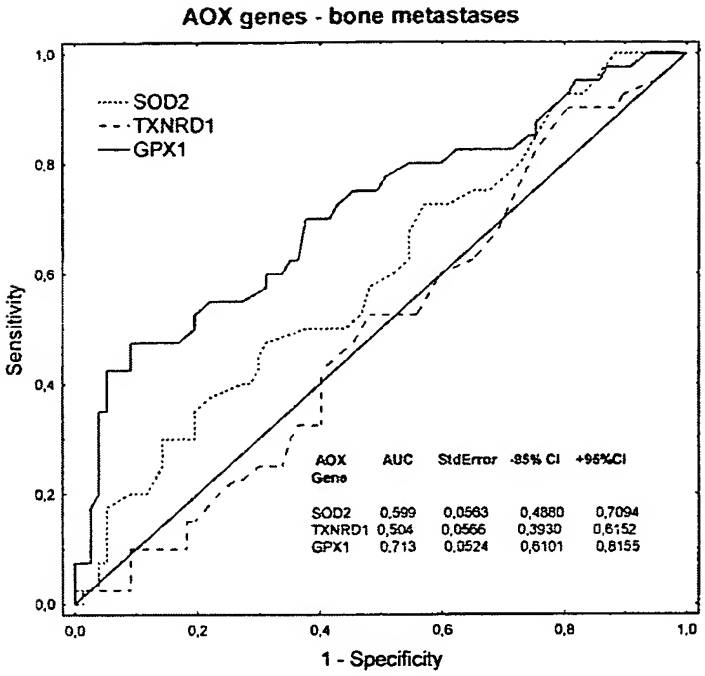
Gene	Sensitivity	Specificity	PPV	NPV	Prevalence	accuracy	odds ratio	p-value
	CCC gene expression (Threshold values: SOD2 = 2.4; GPX1 = 3.4)							
GPX1	71%	65%	57%	77%	40%	67%	4.3	0.0001
SOD2	51%	70%	53%	68%	40%	63%	2.5	0.01
2 genes	75%	49%	49%	75%	44%	65%	2.9	0.007

Figure 5:



Group 1 = no relapse Group 2 = distant relapse	mean value group 1	mean value group 2	p	n (group 1)	n (group 2)
SOD2	2,019481	3,217647	0,006015	77	51
TXNRD1	1,650000	1,645098	0,984745	77	51
GPX1	2,915584	6,007843	<0,000000	77	51

Figure 6:



Spearman's Correlation by Rank (distant vs. no relapse)

	valid - N	Spearman - R	t(N-2)	p-Level
Diagnosis (distant relapse) & SOD2	128	0,242400	2,804581	0,005837
Diagnosis (distant relapse) & TXNRD1	128	0,033932	0,381107	0,703765
Diagnosis (distant relapse) & GPX1	128	0,432834	5,389562	0,000000

Summary: Parameters and Standard Error

Logistic Regression Analysis (distant relapse vs. no relapse)

	Konst.B0	SOD2	TXNRD1	GPX1
Estimate	-1,654726	0,1181642	-0,3712639	0,361583
Standard Error	0,4060642	0,1043102	0,1966556	0,08377139
t(124)	-4,075035	1,132816	-1,887889	4,316307
p-Niveau	0,00008154214	0,2594772	0,06137776	0,00003212131
-95%CL	-2,458441	-0,08829483	-0,7605004	0,195776
+95%CL	-0,851011	0,3246233	0,0179725	0,5273901
Wald's Chi-square	16,60591	1,283272	3,564126	18,63051

p-Niveau	0,00004611836	0,2573	0,05904983	0,00001591373
Odds Ratio (je Einh)	0,1911445	1,125429	0,6898618	1,4356
-95%CL	0,08556828	0,9154909	0,4674325	1,216254
+95%CL	0,426983	1,383509	1,018135	1,694504
Odds Ratio (Range)		5,418118	0,05734141	189,2283
-95%CL		0,2829129	0,002863093	17,09442
+95%CL		103,7634	1,148421	2094,68

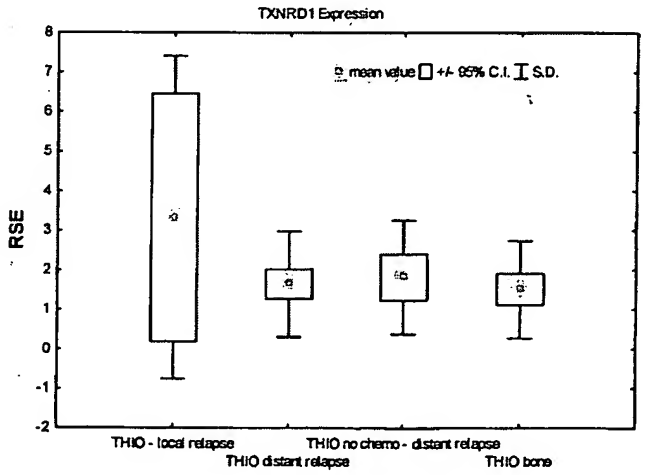
Classification of cases (-no relapse vs. distant relapse-total.)			
Diagnosis	**Progn – no relapse**	**Progn – distant relapse**	**Percent correct**
0,000000	67	10	87,01299
1,000000	21	30	58,82353

The average correct classification value was 75%.

5.3. 2 Local Relapse

A total of 49 patients (40 patients with bone metastases; 9 patients with local relapse only) were enrolled in this assessment.

Figure 7:



t-test	average - local	average - bone	p	valid N - local	valid N - bone
local SOD2 vs. bone SOD2	3,566667	2,652500	0,318894	9	40
local GPX1 vs. bone GPX1	6,022222	5,560000	0,754539	9	40
local-TXNRD1 vs. bone TXNRD1	3,322222	1,512500	0,019314	9	40

Whereas distant relapse was predicted with GPX1 and SOD2, local relapse was predicted with GPX1, SOD2 and TXNRD1. The elevated expression of TXNRD1 in the case of local relapse allowed to differentiate local from distant relapse (figure 7).

Table IV. Performance of gene expression in CCC for the prediction of local prostate relapse

Gene	Sensitivity	Specificity	PPV	NPV	Prevalence	accuracy	odds ratio	p-value
	CCC gene expression (Threshold values: TXNRD1 = 1.6)							
TXNRD1	56%	70%	29%	88%	10%	65%	3	0.1

This was confirmed by logistic regression analysis:

Logistic Regression Analysis (distant relapse vs. local relapse)

	Const.B0	TXNRD1	SOD2	GPX1
Estimate	1,014053	-0,5227636	-0,1258682	0,4586183
Standard Error	0,7398865	0,2543058	0,1609272	0,2285987
t(45)	1,370553	-2,055649	-0,7821441	2,006216
p-Level	0,1773127	0,0456461	0,4382263	0,05086602
-95%CL	-0,4761544	-1,034962	-0,4499922	-0,001803022
+95%CL	2,504261	-0,01056538	0,1982557	0,9190397
Wald's Chi-quadra	1,878415	4,225694	0,6117494	4,024903
p-Level	0,1705241	0,03982442	0,4341356	0,04484158
Odds Ratio (je Einh)	2,756752	0,5928798	0,881731	1,581887
-95%CL	0,6211675	0,35524	0,6376331	0,9981986
+95%CL	12,23452	0,9894902	1,219274	2,506882
Odds Ratio (Range)		0,00318151	0,176051	643,2404
-95%CL		0,00001136946	0,002009454	0,9748978
+95%CL		0,8902801	15,42407	424411,9

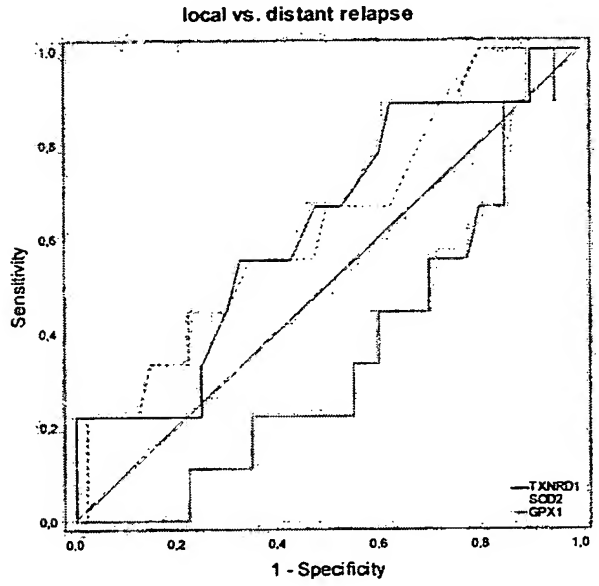
Classification of cases (distant relapse vs. local relapse)

Relapse	Progn - local	Progn - bone	Percent - correct

local	2	7	22,22222
bone	1	39	97,50000

The correct predictive classification of cases was 83%.

Figure 8:



	AUC	StdError	95% Lower CI	95% Upper CI
TXNRD1	0,626	0,1088	0,4132	0,8396
SOD2	0,628	0,1087	0,4147	0,8409
GPX1	0,651	0,1079	0,4399	0,8628

5.4 Summary and conclusion

Prostate cancer was used as the example for the clinical utility of SOD2, TXNRD1 and GPX1 expression level in disseminated cancer cells (CCC: circulating cancer cell clusters) purified from blood through a filtration device (mesh width ~20µm). The clinical utility was assayed for prediction of the primary tumor, distant (bone) and local relapse and for disease prognostication (relapse-free survival). Predicting the primary tumor as well as local relapse involved the evaluation

of all three genes. For disease prognostication (relapse-free survival) as well as for predicting bone metastases SOD2 and GPX1 were the lead markers. In summary, the correctness of prediction amounts to values between >70% and < 90%.

While said studies have been carried out on blood and with respect to prostate cancer, it can reasonably be expected that essentially the same method can be applied to bone marrow and other cancer types.

6. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

7. Further deponent saith not.

Lienen, Germany, 09/12/08 .....